

Lack of homologous sequence-specific DNA methylation in response to stable dsRNA expression in mouse oocytes

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ABSTRACT

Double-stranded RNA (dsRNA) induces sequence-specific mRNA degradation in most eukaryotic organisms via a conserved pathway known as RNA interference (RNAi). Post-transcriptional gene silencing by RNAi is also connected with transcriptional silencing of cognate sequences. In plants, this transcriptional silencing is associated with sequence-specific DNA methylation. To address whether this mechanism operates in mammalian cells, we used bisulfite sequencing to analyze DNA in mouse oocytes constitutively expressing long dsRNA against the *Mos* gene. Our data show that long dsRNA induces efficient *Mos* mRNA knockdown but not CpG and non-CpG DNA methylation of the endogenous *Mos* sequence in oocytes and early embryos. These data demonstrate that dsRNA does not directly induce DNA methylation in the *trans* form of this sequence in these mammalian cells.

INTRODUCTION

Post-transcriptional gene silencing mediated by double-stranded RNA (dsRNA), termed RNA interference (RNAi), is found in most eukaryotes. Analysis of eukaryotic RNAi pathways suggests that RNAi is connected to transcriptional silencing of homologous sequences in certain species. Effects of dsRNA that result in transcriptional silencing include DNA methylation in plants (1–3) and heterochromatin formation in fungi (4,5). A connection between post-transcriptional gene silencing and transcriptional gene silencing in animals is not established, but there are hints that a similar connection exists at the level of chromatin (6,7).

De novo dsRNA-directed DNA methylation (RdDM) has been described only in plants (8), but the mechanism of RdDM in which symmetrical (CpG, CpNpG) and non-symmetrical (CpNpNp) cytosines are methylated is not well understood. Analysis of RdDM mutants indicates a requirement for the DNA methyltransferases MET1 and DDM1 for maintenance of RdDM methylation (8) and *de novo* DNA methylation induced by expression of an inverted repeat does not occur

in *drm1/2* double mutants (9). It is not known whether an RdDM mechanism exists in animals, particularly mammals, in which DNA methylation is one of the most important epigenetic modifications implicated in gene regulation. Despite apparent differences [e.g. plant-specific chromomethylases (10)], DNA methylation in plants and mammals appears conserved. For example, plant DDM1 helicase, which is required for maintenance of DNA methylation, is a homolog of the mammalian LSH gene, which is required for maintenance methylation (11); there is a partial sequence similarity between plant DRM1/2 *de novo* DNA methylases and the animal Dnmt3 family of *de novo* DNA methyltransferases (12). In addition, it has been speculated that non-CpG methylation, which is present in early mouse embryos (13), is directed by RNAi (8). These data suggest a common ancestry of this epigenetic regulation in plants and mammals and provide the rationale for determining whether RdDM occurs in mammalian cells.

Mouse oocytes are a suitable model system to analyze whether RdDM operates in mammals. The RNAi pathway functions in oocytes (14–16), and nuclear expression of dsRNA during oocyte development, induces an RNAi effect (17–19). Oocytes exhibit *de novo* CpG methylation of imprinted genes (20–22), which requires Dnmt3l DNA methyltransferase (23) that is expressed in growing oocytes along with Dnmt1, 3a, and 3b (24). DNA methylation is also implicated in silencing of repetitive mobile elements, which are methylated in the germline (25,26). Endogenous retrovirus IAP (Intracisternal A particle) is an example of such a mobile repetitive element, which is constrained by DNA methylation (27) and methylated *de novo* in growing postnatal oocytes (22).

Notably, many imprinted regions exhibit overlapping sense and antisense transcripts (28–31) and repetitive elements can likely generate dsRNA (32,33). In fact, IAP dsRNA has been isolated from mammalian cells (32) and IAP transcripts are likely recognized by RNAi in preimplantation mouse embryos (34). Thus, there is clearly the potential to generate the dsRNA essential to trigger the RNAi pathway that could modulate the expression of these genes.

We have previously developed a transgenic RNAi approach to study gene function in oocyte development. This approach also allows assessment of a link between RNAi and DNA methylation in the mouse female germ cells (Figure 1). This model system employs the endogenous *Mos* gene as a

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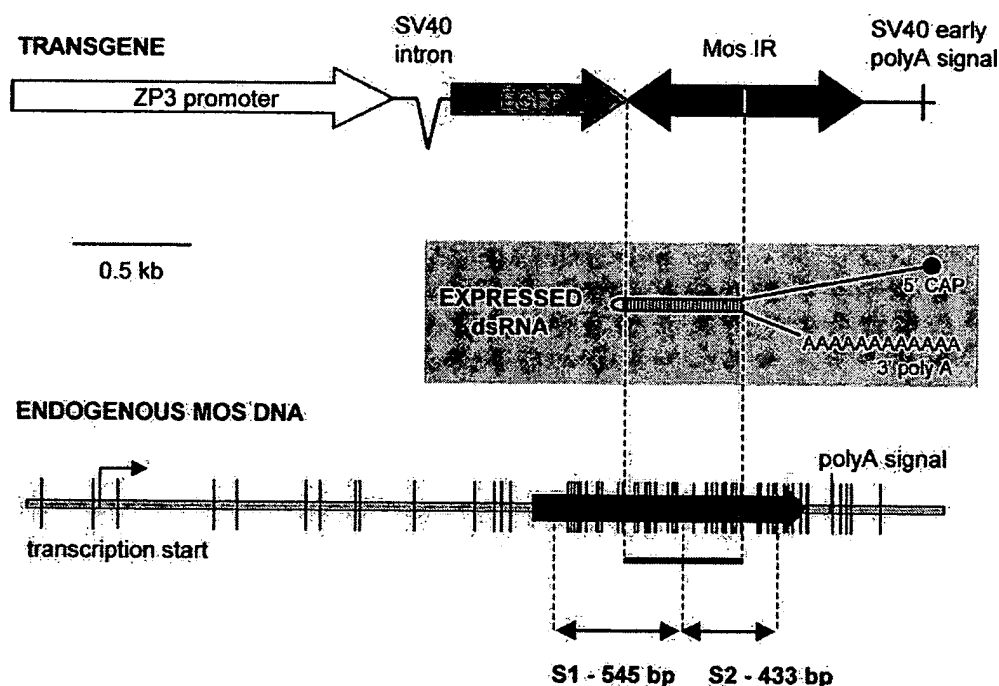


Figure 1. Schematic description of the model system. The transgene [described in detail in (15,17)] expresses a dsRNA hairpin. The length of the expressed dsRNA is ~0.5 kb and this RNA is presumably capped and polyadenylated. The bottom shows the position of the homologous sequence and the amplified sequence in the *Mos* gene (*Mos* is an intronless gene). EGFP, enhanced green fluorescent protein; IR, inverted repeat. The coding sequence is red. The schematic diagram of the *Mos* gene is drawn to scale.

target for *Mos* dsRNA, which is expressed as a long dsRNA hairpin; expression is controlled by the *Zp3* promoter, an oocyte-specific promoter that is active in growing oocytes (35). Using this model system, we previously documented that a *Zp3*-driven transgene expressing *Mos* dsRNA induces sequence-specific *Mos* mRNA decrease in oocytes of living animals (17).

Here we report results of the DNA methylation analysis of the endogenous *Mos* sequence homologous to the dsRNA expressed during oocyte development. Our results suggest that dsRNA expression, while inducing post-transcriptional silencing, does not induce sequence-specific DNA methylation of the cognate DNA sequence.

RESULTS AND DISCUSSION

To examine DNA methylation of the endogenous *Mos* sequence, we isolated oocytes from transgenic mice expressing *Mos* dsRNA and mapped the DNA methylation pattern in the corresponding region of the endogenous *Mos* gene by bisulfite mutagenesis. The *Zp3* promoter functions from the onset of oocyte growth (36), which takes ~3 weeks, and hence the siRNAs produced from the transgene should, in principle, have ample time to elicit sequence-specific DNA methylation. Bisulfite treatment results in the conversion of non-methylated cytosine residues to uracil residues, which appear as C-T conversion after PCR amplification of treated DNA. Because methylated cytosines are not converted, sequencing of cloned PCR products allows identification of individual methylated cytosines of individual DNA molecules.

We used F2 females produced by transgenic F1 males obtained from the male H13 founder line carrying the *Mos* RNAi transgene, which induces a strong RNAi effect in oocytes [described in detail in (17)]. We confirmed that RNAi functioned in oocytes from transgenic females that were used for the DNA methylation analyses by demonstrating that MAP kinase activity was markedly reduced following maturation of the transgenic oocytes (16) (data not shown).

We analyzed the cognate sequence, as well as adjacent sequences, in transgenic oocytes with two pairs of primers (S1 and S2 PCR products, Figure 1); oocytes were collected from four transgenic mice. This permitted comparison of DNA methylation of the cognate and neighboring sequences within one amplicon (PCR product). Bisulfite sequencing was also performed on DNA from non-transgenic oocytes, and DNA from somatic cells (tail DNA), in which the transgene either was not present (wild type) or should not be expressed (transgenic). These samples served as controls. More clones were obtained for the S1 primer pair because it worked more efficiently (Figure 2). For the S1 primer set, each sample produced at least two independent PCR products (usually 4), and we identified a total of 19 unique sequences from transgenic oocytes. Similarly, we analyzed oocytes from 3 wild-type animals (16 unique sequences), tail DNA samples from 4 wild-type animals (25 unique sequences) and 3 transgenic animals (31 unique sequences).

To classify sequenced clones as unique we used the following criteria: Two clones originating from different PCR reactions were considered unique by definition (they had to be

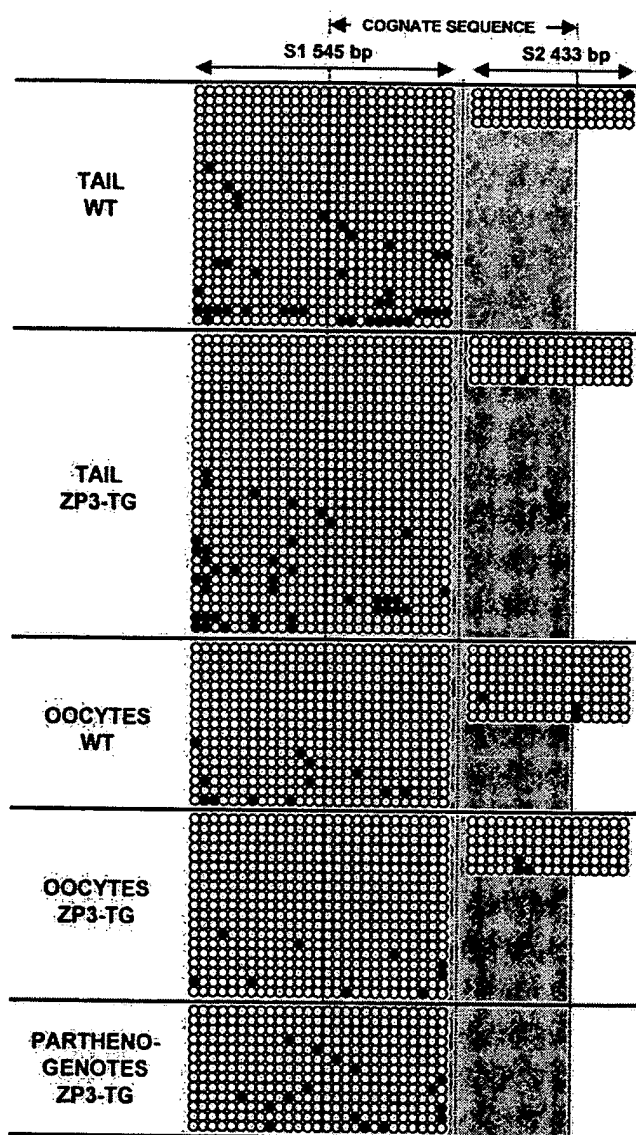


Figure 2. Bisulfite sequencing of the *Mos* gene. Open circles represent non-methylated cytosines in CpGs in the analyzed sequence, and the filled circles represent methylated cytosines. Rows of circles represent unique DNA molecules as described in the text. The gray area depicts CpGs within the cognate sequence. ZP3-TG, transgenic; WT, wild type.

amplified from different templates). Clones derived from the same PCR reaction with different methylation patterns were also considered unique. Clones derived from the same PCR reaction with the same methylation pattern (or absence of methylation) were considered the same unless they showed a different pattern of non-converted cytosines.

It is possible that non-converted cytosines actually represent T→C mutations introduced during PCR and cloning. The probability that a non-CpG cytosine originates from incomplete bisulfite conversion, however, is higher than that it originates from the T/C mutation. Detailed analysis of all sequences from the upstream region revealed that the observed T/C mutation frequency of original T residues was $\sim 1.6 \times 10^{-3}$

while the observed frequency of non-CpG cytosines was 3 times higher. Therefore, we used 'non-converted cytosines' as an additional criterion, especially in cases where two unmethylated clones were found in the same amplicon. This decision was supported by the fact that we saw only once two clones from one amplicon with an identical methylation pattern, which differed in the 'non-converted' cytosine residue. It should be noted that dsRNA expression did not affect non-CpG methylation, which is also detected by bisulfite sequencing. As mentioned above, non-CpG cytosines found in this experiment may also stem from T/C mutations and incomplete bisulfite mutagenesis. Unconverted non-CpG cytosines appeared with a frequency of approximately 0.5 per 100 non-CpG cytosines and there was no apparent difference between analyzed samples (Figure 3A).

Analysis of the DNA methylation patterns did not reveal any marked increase in DNA methylation in the cognate sequence region of the endogenous *Mos* gene in transgenic oocytes, when compared to the set of controls (Figures 2 and 3B). Thus, it appeared unlikely that an RNAi-mediated sequence-specific DNA methylation functions in mouse oocytes and could serve as a means of modulating gene expression. Nevertheless, it was possible that DNA replication is required for sequence-specific DNA methylation, and oocytes grow while arrested in the first meiotic prophase, i.e. there is no DNA replication. Of note is that replicating plant cells were used in the reported RdDM studies, and no information exists if sequence-specific DNA methylation occurs in non-replicating plant cells.

To address this issue, we exploited the *Mos* knockdown phenotype, which is parthenogenetic activation, and the ensuing DNA replication. Accordingly, we analyzed DNA from parthenogenetic two- and four-cell embryos derived from transgenic oocytes that matured *in vitro* and then underwent activation. The RNAi effect likely continues to persist at this time, because CTCF expression is not observed in embryos derived from CTCF knockdown transgenic oocytes [(18) and P. Svoboda, unpublished results]. Bisulfite mutagenesis of DNA obtained from three-day old parthenogenotes did reveal a slight increase in sequence-specific DNA methylation of the cognate *Mos* gene sequence when parthenogenotes were compared to transgenic and non-transgenic oocytes (Figures 2 and 3B). In fact, parthenogenotes exhibit a statistically significant increase when the frequency of CpG methylation in the cognate sequence is compared to that of the WT or transgenic oocyte (one-tailed T-test, $p < 0.05$). This increase, however, is not statistically significant when compared to DNA methylation levels in any upstream sequence (including its own) or cognate sequences in tail DNA from WT and transgenic animals. In addition, when total DNA methylation levels of S1 amplicons are compared, parthenogenotes significantly differ only from transgenic oocytes (Figure 3B). The statistical analysis, however, was markedly influenced by a single DNA clone from WT oocytes, which exhibits 4 methylated cytosines (one-third of all methylated cytosines in 13 sequences from WT oocytes; Figure 2). Excluding this clone would increase the difference between parthenogenotes and WT oocytes (Figure 3B, compare OO WT and OO WT* samples with the PG ZP3 sample).

Several conclusions can be drawn from these studies. First, the similar levels of DNA methylation in upstream and cognate sequences of transgenic parthenogenotes argue against a

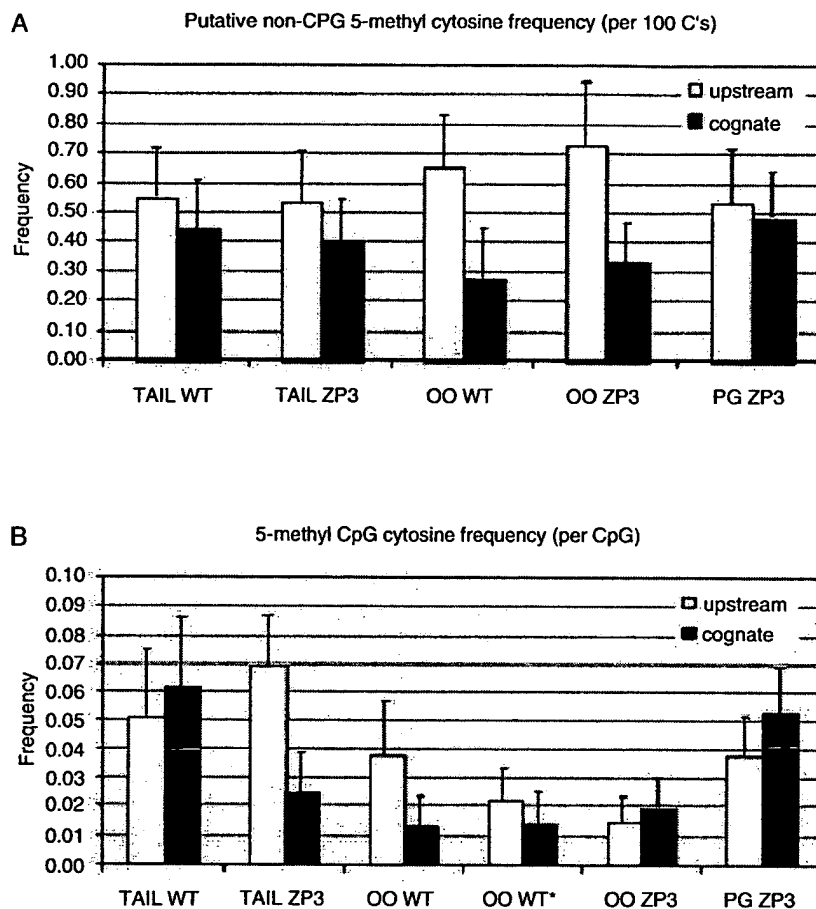


Figure 3. Quantification of bisulfite sequencing results. (A) Relative abundance of non-CpG cytosines per 100 cytosines in upstream and cognate sequences in the S1 amplicon. Black bars, cognate sequence (256 nt of the amplicon, 63 non-CpG cytosines); white bars, upstream sequence (289 nt, 72 non-CpG cytosines). The data are expressed as the mean \pm SEM. (B) Normalized frequency of methylated cytosines in the cognate sequence and upstream. The total number of methylated CpGs in a given region of a given sample was divided by the number of individual clones multiplied by the number of CpGs in the region. The resulting number thus represents the average DNA methylation frequency per CpG in the given region. The data are expressed as the mean \pm SEM. TAIL WT, tail samples of wild-type animals; TAIL ZP3, tail samples from transgenic animals; OO WT, wild-type oocytes; OO WT*, same as previous sample, except one DNA clone with four cytosines was omitted for the analysis to demonstrate its effect on the outcome of the experiment; OO ZP3, oocytes from transgenic RNAi animals, in which dsRNA is expressed, PG ZP3, parthenogenotes obtained from spontaneous parthenogenetic activation of matured oocytes from transgenic animals.

direct dsRNA (or derived siRNAs)-directed DNA methylation, which should affect only the cognate sequence. If dsRNA expression affects DNA methylation of homologous sequences in parthenogenotes at all, the effect is likely secondary and probably mediated by changes in chromatin structure. This possibility cannot be addressed in our model system due to the limited amounts of obtainable material. Second, the absolute level of CpG methylation of cognate sequences is $\sim 5\%$ in transgenic parthenogenotes and $\sim 2\%$ in transgenic oocytes. These levels are comparable to background DNA methylation levels observed in non-transgenic DNA samples and therefore likely to be biologically insignificant. This hypomethylation contrasts with levels of *de novo* methylation of imprinted genes and IAP in normal growing oocytes (22) or with methylation changes of the differentially methylated domain (DMD) of *H19* in mouse oocytes in a CTCF knock-down experiment (18). The CTCF knockdown is mediated by the same transgenic RNAi design and the absence of the CTCF correlates with strong hypermethylation of the *H19* DMD in

several bisulfite-sequenced clones. This hypermethylation occurred within the same time window used in our experiment. Third, dsRNA expression in oocytes was driven by the strong *Zp3* promoter and *Mos* dsRNA levels are very likely higher than for other dsRNAs in the oocyte. Thus, RdDM hypermethylation of sequences that can generate dsRNA (e.g. the repetitive mobile element IAP) is unlikely to be directly controlled in *trans* by dsRNA in oocytes and preimplantation embryos. Whether dsRNA could affect chromatin structure in homologous DNA sequences remains an open question.

It would be premature to conclude that RdDM is absent in mammals. Our model system is confined to a specific cell type and we studied RdDM targeting in a single intronless endogenous gene. Unfortunately, we were not able to conduct similar experiments with *Ctcf* and *Msy2*, the other two available transgenic RNAi models (18, 19), because RNAi-targeted sequences of these two genes are normally hypermethylated in oocytes (data not shown). Because data from plants suggests that there is a difference in efficient RdDM between transgenes

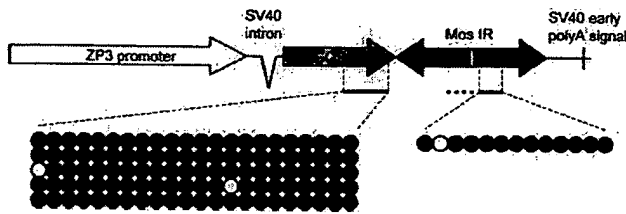


Figure 4. Bisulfite sequencing of the RNAi transgene in tail DNA. The RNAi transgene is methylated even if it is not active. Tail sample, in which the *Zp3* promoter is not active, was obtained from one of the transgenic females used for analysis of *Mos* methylation in transgenic oocytes (Figure 2). Open circles represent non-methylated cytosines in CpGs in the analyzed sequence, and the filled circles represent methylated cytosines. Rows of circles represent unique DNA molecules as described in the text. The *Mos* PCR amplified the central part of the inverted repeat (dotted line plus full line) but only the first 200 nt (full line) could be sequenced due to the nature of the template. EGFP, enhanced green fluorescent protein; IR, inverted repeat.

and endogenous genes (37), future experiments are needed, which will address RdDM using various target sequences in different mammalian cells.

Last, our investigation addressed RdDM of homologous sequences in *trans* because such RNAi-related mechanism occurs in plants. Nevertheless, dsRNA could also function in *cis*. DNA methylation in *cis*-linked to antisense RNA transcription (which could form dsRNA) occurs in human cells (38). We analyzed DNA methylation of the *Mos* RNAi transgene (Figure 4). We were not able to amplify the inverted repeat from oocyte DNA, presumably because the single-stranded DNA forms a double-stranded hairpin, which does not allow efficient bisulfite mutagenesis, but one hypermethylated clone derived from the inverted repeat was obtained after repeated bisulfite sequencing of tail DNA of one of the transgenic animals (Figure 4). It is unlikely that this hypermethylation is related to dsRNA expression because the *Zp3* promoter is active only in oocytes and the transgene was always maintained in the paternal lineage. Thus, the transgene was never expressed except in oocytes of the analyzed animal. In addition, the upstream EGFP sequence in the same DNA is hypermethylated as well, further suggesting that methylation of the transgene with the inverted repeat is dsRNA independent.

MATERIALS AND METHODS

Bisulfite sequencing of DNA from oocytes

Twenty five to fifty oocytes (or parthenogenotes) were isolated from each transgenic or non-transgenic female. Oocytes from one animal were embedded in low melting agarose (FMC, final volume and concentration ~20 μ l and 1.6%, respectively). Five oocytes were set aside and used for the MAP kinase assay (16). The agarose beads were treated in lysis buffer (800 μ l of 10 mM Tris-HCl, pH 7.6, 10 mM EDTA, 1% SDS, 50 μ g/ml proteinase K) at 50°C overnight. The beads were washed 3 times with 1 ml of TE 10/1 (10 mM Tris-HCl, pH 7.6, 1 mM EDTA) and DNA was denatured in 500 μ l of 0.3 M NaOH twice for 15 min each time. Beads were solidified in 500 μ l of 0.1 M NaOH for 10 min, transferred to a tube, melted at 80°C for 10 min, and solidified on ice. Bisulfite treatment (with 10 μ l of 10 mM hydroquinone and 255 μ l of 40.5% sodium bisulfite, freshly prepared) was performed

in the dark at 50°C for 6 h. After bisulfite treatment, beads were washed 6 times with 1 ml of TE 10/1 and then treated twice for 15 min each time with 500 μ l of 0.2 M NaOH (second treatment was at 37°C). A 100 μ l of 1 M HCl was added to neutralize the NaOH at the end of the second NaOH treatment. Beads were briefly washed in 1 ml of TE 10/1, washed twice with MilliQ H₂O (1 ml, 15 min each) and then melted with H₂O to obtain 100 μ l of diluted melted agarose. Melted agarose was immediately used as templates for PCR (Amplitaq Gold, Perkin Elmer, 100 nM primers, 25 μ l of melted agarose per reaction, volume 100 μ l). S1 primers: sense, 5'-TATGT-GATTGTTTTATTTGAGGGTGTAAT-3'; antisense, 5'-CCC-AAACTCAATTATTCTCTACAACTAAAA-3'. S2 primers: sense, 5'-GAAGTGTTTTAAGTATTTTTTAGATGTTGT-TAA-3'; antisense, 5'-CAACAACCTCTAAATAATATTCTA-CAATATCTTTC-3'. EGFP primers: sense, 5'-TAACCATAC-TTATCTACCTCCAAATAAATA-3'; antisense, 5'-GTA-TAGTTCGTTTAT GTCGAGAGTGATT-3'. *Mos* hairpin primers: S1 antisense and 5'-AATTTAGTTGTTTTTG-TAGTTGAGAGGTTT-3'. The PCR program consisted of 15 min of initial denaturation at 94°C followed by 20 cycles of touchdown PCR using T_m from 62 to 52°C (decrease by 0.5°C per cycle) followed by 35 cycles at T_m 55°C. Denaturation steps in each cycle were performed at 94°C for 30 s, annealing steps with temperatures shown above (T_m) for 30 s and all extension steps at 72°C for 1 min. A one-extension step was added at 72°C for 15 min at the end of the program to facilitate TOPO-TA cloning. PCR products were resolved in a 1.2% agarose gel. Amplicons were gel extracted (Qiagen Gel Extraction Kit) and cloned by using the TOPO-TA cloning kit (Invitrogen). At least four clones from each cloned amplicon were selected for DNA isolation and sequencing.

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